Antioxidant capacity in fruit of Citrus cultivars with marked differences in pulp coloration: contribution of carotenoids and vitamin C

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ABSTRACT

The purpose of this study was to evaluate the specific contribution of carotenoids and vitamin C to the lipophilic and hydrophilic antioxidant capacity (LAC and HAC), respectively, of the pulp of citrus fruits using the genetic diversity in pigmentation and in the carotenoid complement. To this end six citrus varieties were selected: two mandarins, Clemenules (*Citrus clementina*) and Nadorcott (*Citrus reticulata*); two grapefruits (*Citrus paradisi*), Marsh and Star Ruby; and two sweet oranges (*Citrus sinensis*), Valencia late and Valencia Ruby. Total carotenoid content and composition in the pulp of fruits were very different, in relation to their color singularities. Valencia Ruby and Nadorcott had the highest carotenoid content, accumulating the former large amounts of linear carotenes (phytoene, phytofluene and lycopene) and Nadorcott of β-cryptoxanthin. Orange fruits contained the highest amount of vitamin C while in Nadorcott mandarin was more that 50% lower. Analysis of antioxidant capacity, evaluated by ABTS and DPPH assays, in the pulp of the different fruit varieties indicated a high and positive correlation between vitamin C content and hydrophilic antioxidant capacity (HAC). Nevertheless, a weak correlation was observed between carotenoids content and lipophilic antioxidant capacity (LAC) in the pulp extracts assayed by ABTS. Overall, vitamin C in the pulp of citrus fruit had an important contribution to the HAC, whereas that of carotenoids to LAC was very variable; being the highest that of Valencia Ruby orange, with large concentrations of lycopene and phytoene, followed by Nadorcott mandarin, with high β-cryptoxanthin content.

Keywords: Antioxidant capacity, Carotenoids, Citrus fruit, Vitamin C

INTRODUCTION

Citrus fruit are one of the primary fruit crops in international trade in terms of value, and are highly demanded worldwide for fresh consumption, juice processing, freeze-concentrates and also as food additives for dishes and beverages (Talón et al., 2020). Color is one of the most remarkable features among the different Citrus species and cultivars and a key factor of external and internal quality and consumer's acceptance. The genus *Citrus* shows a high diversity in external and internal fruit coloration: from the yellow color of lemons (*Citrus limon*), pummelos (*Citrus maxima*), many grapefruits cultivars (*Citrus paradisi*) and citrons (*Citrus medica*), to the orange of mandarins (*Citrus reticulata, C. clementina* or *C. unshiu*) and sweet oranges (*Citrus sinensis),* and red or pink in some lycopene-accumulating grapefruits or new sweet orange cultivars (Liu et al., 2007; Alquézar et al., 2008; Lu et al., 2017).

Citrus fruit are good sources of useful phytochemicals, such as vitamins A, C and E, mineral elements, flavonoids, phenolic acids, coumarins, limonoids, anthocyanins (blood oranges), carotenoids and pectins, among others (Lv et al., 2015; Patil et al., 2017; Zou et al., 2016). Evidence from several *in vitro* and *in vivo* studies have related the consumption of citrus fruit with important health benefits and the reduction in the risk of chronic diseases (Silveira et al., 2015; De Oliveira et al., 2019; Ma et al., 2020; Llopis et al., 2019; Jing et al., 2020). The beneficial effects of these compounds in human health have been partially associated with their antioxidant activity (Barros et al., 2018; Rajendran et al., 2014). In general, antioxidants are natural or synthetic compounds that may prevent or delay the oxidative cell damage caused by physiological oxidants, scavenging or quenching reactive oxygen (ROS) or nitrogen species (RNS), products of respiration, including free radicals (Barros et al., 2018).

Carotenoids are among the most important phytochemicals in citrus fruit. They are lipophilic pigments responsible for the coloration of mature fruit in most *Citrus* species and cultivars, and therefore, their content and composition have a strong impact on their commercial acceptability (Gross et al., 1987; Tadeo et al., 2020). Citrus fruit are one of the most complex sources of carotenoids with a large diversity of them among the different species and cultivars in terms of types and amounts (Kato, 2012; Rodrigo et al., 2013a). Carotenoid content and profile are influenced by genotype and, in general, mandarin and orange cultivars accumulate larger concentrations of carotenoids than grapefruits, pummelos and lemons (Kato et al., 2004; Alquézar et al., 2008; Rodrigo et al., 2013a).

In citrus fruit, the antioxidant activity mainly depends on a complex combination of hydrophilic (e.g., ascorbic acid and polyphenols) and lipophilic compounds (e.g., carotenoids and tocopherols) (reviewed by Zou et al., 2016). In plants, carotenoids play important roles

protecting biological systems against photooxidative processes and oxidative damage, as they are efficient antioxidants scavenging singlet molecular oxygen ($^1O_2$) and peroxyl radicals (Stahl and Sies, 2003; Müller et al., 2011). The efficiency of carotenoids for physical quenching is related to the number of conjugated double bonds of the molecule, being lycopene the most efficient carotenoid with eleven (Di Mascio et al., 1989; Stahl and Sies, 2003). Furthermore, the presence of ionone rings, hydroxyl, epoxy or keto functional groups, modulates the ability of carotenoids to scavenge free radicals (Di Mascio et al., 1989; Stahl., 2003; Müller et al., 2011; Apak et al., 2013). A large number of studies have analyzed over the years the antioxidant activities of citrus fruit extracts, focusing on the antioxidant assessment by a single extraction of the bioactive compounds in specific solvents, irrespectively of the selective solubility of each family of compound in the extraction mixture (Cano et al., 2004). The general conclusions emerging from several works suggest positive correlations between total antioxidant activity and vitamin C content and phenolic compounds in pulp and juice of different *Citrus* species and cultivars (Franke et al., 2004; Rapisarda et al., 2008; Gironés-Vilaplana et al., 2014; Yoo and Moon, 2016; Sicari et al., 2016; De Ancos et al., 2017, 2020). Despite the well-recognized antioxidant capacity of some carotenoids, their relative contribution to the total antioxidant capacity of foodstuff is still controversial (Rodríguez-Amaya, 2010). Different studies indicated that carotenoids contribute to the antioxidant capacity of *Citrus* (Sánchez-Moreno et al., 2003; De Ancos et al., 2020). Sánchez-Moreno et al. (2003) reported a positive correlation between β-cryptoxanthin content and the antioxidant capacity of orange juices but a lack of correlation with other carotenoids. However, other works have not found a relationship between carotenoid content and the antioxidant capacity (De Ancos et al., 2002), or lower than that of phenolic compounds or vitamin C (Gardner et al., 2000; Sánchez-Moreno et al., 2003; Cano et al., 2004; Yoo and Moon, 2016). It seems that the extraction solvents and the antioxidant assay may be critical factors for the discrepancy among the different studies (Sánchez-Moreno et al. 2003; Rodríguez-Amaya et al., 2010; Müller et al., 2011). Moreover, the evaluation of the antioxidant capacity of both the water-soluble and lipid-soluble fractions of citrus fruit extracts has been scarcely studied (Cano et al., 2004).

The aim of this study was to use the genetic diversity in coloration and carotenoid composition of citrus fruits to evaluate the antioxidant capacity of the lipophilic (LAC) and hydrophilic (HAC) fraction independently, through the scavenging DPPH and ABTS radical assays. To that end, we have used fruits of two genotypes of oranges, two grapefruits and two mandarins with contrasting differences in pulp coloration, and determined carotenoids content and composition, as well as vitamin C content (Vit C), and analyzed their contribution to the lipophilic and hydrophilic antioxidant capacity, respectively.

MATERIAL AND METHODS

Plant Material

Fruits of two genotypes of sweet orange (*Citrus sinensis*), Valencia late and Valencia Ruby, two grapefruits (*Citrus paradisi*) Marsh and Star Ruby and two mandarins Clemenules (*Citrus clementina*) and Nadorcott (*Citrus reticulata*), with contrasting differences in pulp coloration were selected (Figure 1). Fruits of each genotype were harvested from adult trees developed under standard agronomical and growing conditions. Fruits were harvested at commercial maturity, and dates and growing locations were as follows: January and February for Clemenules and Nadorcott mandarins, respectively, from a commercial orchard located in Lliria (Valencia, Spain); both grapefruits were harvested in February at The Citrus Germplasm Bank from the Instituto Valenciano de Investigaciones Agrarias (IVIA, Moncada, Valencia, Spain), and the late-harvesting Valencia late and Valencia Ruby oranges were harvested in April from the Fundación ANECOOP (Museros, Valencia, Spain). At least 60 fruits for each genotype were harvested and immediately delivered to the laboratory. Fruits were selected for size uniformity and free of any external damage or defect. Fruits were sliced into halves and after determination of pulp color, pulp tissue was obtained by excising small cube pieces of approximately 1 cm^2 containing juice vesicles free of segment membranes, immediately frozen in liquid nitrogen and stored at -80 °C until analysis. Juice was extracted from the remaining pulp with a household electric hand reamer (Citromatic MPZ22, Braun, Barcelona, Spain), filtered through a metal sieve with a pore size of 0.8 mm, immediately frozen in liquid nitrogen and stored at −80 °C until analysis.

Color and internal maturity index determinations

Color of the pulp was measured using a CR-400 Minolta chromameter on three different positions. Hunter parameters *a* (negative to positive corresponds from green to red) and *b* (negative to positive, from blue to yellow) were determined, and color was expressed as the a/b Hunter ratio, a classical relationship for color measurement in citrus fruit (Stewart and Wheaton, 1972). Data of color index for each cultivar are the means ± SD of at least 10 fruits.

Total soluble solids (TSS) and total titratable acidity (TA) of the juices were determined using a digital refractometer PAL-BX/ACID1 (ATAGO, Japan). TSS is expressed as °BRIX and TA as mg of citric acid/100 mL of juice and maturity index (MI) was calculated as the TSS/TA ratio.

Carotenoid extraction and analysis by HPLC-DAD

Carotenoids were extracted and analyzed essentially as described by Rodrigo et al. (2015). Briefly, freeze-ground pulp (2 g) was weighed in screw-capped polypropylene tubes, 4 mL of methanol (MeOH) plus 5 mL Tris-HCl (50 mM, pH 7.5) (containing 1 M NaCl) were added and sample was sonicated 5 min in XUBA3 ultrasonic water bath (Grant Instruments, England) at room temperature. Dichloromethane (DCM) (10 mL) was added to the mixture, stirred for 5 min at 4°C and centrifuged at 3000 g for 10 min at 4°C. The hypophase was recovered and the aqueous phase re-extracted with DCM until it was colorless. The pooled DCM extracts were dried on a rotatory evaporator at 40°C. Samples were saponified in methanolic KOH (6%, w/v) overnight. Saponified carotenoids were recovered from the upper phase after adding water and petroleum ether:diethyl ether (9:1) to the mixture. Extracts were dried and kept at −20 °C until further analysis. Each sample was extracted at least twice and results are mean ± SD.

The carotenoid composition of each sample was analyzed by HPLC with a Waters liquid chromatography system equipped with a 600E pump and a photodiode array detector (DAD) model 2998, and Empower3 software (Waters, Spain). A C30 carotenoid column (250 × 4.6 mm, 5 µm) coupled to a C30 guard column (20 × 4.0 mm, 5 µm) (YMC, Teknokroma, Spain) was used. Chromatographic conditions used are described in Lado et al. (2015) and Rodrigo et al. (2015). The carotenoids were identified by absorbance spectra and retention time, peaks integrated at their individual maximal wavelength, and their contents were calculated using the appropriate calibration curves, as described elsewhere (Lado et al., 2015; Rodrigo et al., 2015).

Ascorbic acid determination

Ascorbic acid was extracted and determined essentially as described in Alós et al. (2014). Briefly, pulp tissue (0.5 g) were homogenized for 1 min using a Polytron PT-1035 GT (Kinematica AG) homogenizer with 0.1 % metaphosphoric acid (4 ml). The homogenate was centrifuged for 10 min at 4000 g at 4 °C. The supernatant was filtered through a C18 cartridge (SepPak, Waters, Spain), previously activated with 4 ml of MeOH, 4 ml of MilliQ water and 4 ml of 2 % metaphosphoric acid. The extract was subsequently filtered through a 0.45 µm nylon filter (25 mm diameter, Análisis Vínicos, Spain). The filtrate was directly injected in the HPLC-DAD for ascorbic acid determination. Dehydroascorbic acid (DHA) content was calculated from the difference between total vitamin C and the ascorbic acid contents. To determine total vitamin C, we adapted the protocol described by Alós et al. (2014). Thus, a 200 µl aliquot of the above-mentioned filtrate was incubated for 15 min at room temperature with 100 µl 200 mM DTT in 400 mM Tris-base. Then, the reaction was stopped by acidification with 100 µl of 8.5 % ortho-phosphoric acid.

Sample analysis by HPLC was carried out using a HPLC system Waters ACQ Arc SysCore with DAD and Empower 3 software, using an Ultrabase C18 column (100 × 4.6 mm, 2.5 µm) (Análisis Vínicos, Spain) and a mobile phase of MeOH:water pH 2.5 (adjusted with metaphosphoric acid, 15:85, v/v), 0.2 ml min^{-1} flow and injection volume 10 µl. The temperature of the column was set at 35 °C.

Determination of antioxidant capacity: ABTS and DPPH assays

The 2,2'-azino-di-(3-ethylbenzthiazoline sulfonate) (ABTS) assay was employed to determine antioxidant capacity. The method is based on the capacity of different components to scavenge the ABTS radical cation ($ABTS^{\bullet+}$) compared to a standard antioxidant (Trolox). Total antioxidant capacity (TAC) was quantified as described Legua et al. (2011) with slight modifications, which enables to determine antioxidant capacity due to both hydrophilic and lipophilic compounds in the same extraction. Briefly, for each sample, 1.5 g of pulp were homogenized with Polytron PT-1035 GT (Kinematica AG) in 12 ml of 50 mM phosphate buffer pH 7.8 and 12 ml of ethyl acetate, and then centrifuged at 4000 g for 15 min at 4°C. The upper fraction was used to determine the antioxidant activity due to lipophilic compounds (LAC) and the lower phase to hydrophilic compounds (HAC). In both cases, antioxidant capacity was determined in duplicate in each extract using the enzymatic system composed of ABTS, the horseradish peroxidase enzyme (Sigma, Madrid, Spain) and the oxidant substrate (hydrogen peroxide) in which $ABTS^{\bullet+}$ radicals are generated. For HAC, a reaction mixture containing 10 mM ABTS, H_2O_2 (30%) and 10 mM peroxidase in 120 mM glycine buffer solution (pH 4.5) in a total volume of 285 µl. For LAC, the same reaction mixture in pure ethanol was used, in a total volume of 250 µl. Then, 15 µl of the aqueous phase and 50 µl of the organic phase were added to the reaction medium for HAC and LAC determination, respectively, and the decrease in absorbance which is proportional to the ABTS quenched, was determined after 5 min. The absorbance change of the mixture was determined in a UV/Vis microplate spectrophotometer (Fluostar Omega, BMG Labtech) monitored at 730 nm. A calibration curve was performed with Trolox ((R)-(+)-6-hydroxy-2,5,7,8-tetramethyl-croman-2-carboxylic acid) in the range from 1 to 25 mmol (Sigma, Madrid, Spain), and results are expressed as Trolox equivalent antioxidant capacity (TEAC) per fresh weight of fruit pulp (mg 100 g^{-1}).

The hydrophilic antioxidant activity was also determined using DPPH free radical assay (2,2-diphenyl-1-picrylhydrazyl) as described by Girennavar et al. (2007), with slight modifications. Samples of 0.3 g of pulp were homogenized for 1 min using a Polytron PT-1035 GT (Kinematica AG) homogenizer in 6 ml of MeOH:water (80:20, v/v). The extract was centrifuged for 10 min at 4000 g at 4 °C and the supernatant collected into a new a tube. In a

96-well plate 10 µl of each sample was mixed with 290 µl of 100 mM DPPH solution (in 80% MeOH) and incubated at room temperature in darkness for 30 min. Thereafter, the absorbance change of the mixture was determined in a UV/Vis microplate spectrophotometer (Multiskan FC, Thermo Scientific) at 517 nm. Methanol (80%) was used as a control (10 µl MeOH 80% + 290 µl of DPPH) and each reaction was replicated in three wells. The assay was replicated twice and a curve of Trolox solution with concentrations ranging from 25 to 200 µg ml^{-1} was used as a standard. Absorbance was measured at 515 nm and compared to the absorbance obtained for the control (blank without samples). DPPH scavenging capacity was expressed as inhibition percentages by the formula:

$$\% \text{ DPPH scavenging capacity} = [({}^{515nm}A_{control} - {}^{515nm}A_{sample})/ {}^{515nm}A_{control}] \times 100$$

Contribution of vitamin C and carotenoids to antioxidant capacity

Müller et al. (2011) determined the relative antioxidant capacity (RAC) of several bioactive compounds as equivalents of Trolox. Taking these values as a reference, we have determined the contribution of the carotenoids and vitamin C present in the lipophilic and hydrophilic extracts of the citrus samples to the antioxidant capacity. The RAC value of the carotenoids were: lycopene, 3.9; β-cryptoxanthin, 3.2; β-carotene, 3.1; anteraxanthin, 2; zeaxanthin, 1.9; violaxanthin, 1.6; and phytoene and phytofluene, 1. The RAC of Vitamin C is 1.0, which means that Trolox and vitamin C have the same antioxidant activity (Arnao et al., 2001; Cano et al., 2000). Thus, after determination of the HAC and LAC (as Trolox equivalents) and the concentration of the different compounds, their relative contribution to the antioxidant capacity was calculated.

Statistical analysis

Statistical significance was calculated by one-way analysis of variance (ANOVA) by XLSTAT software and the Tukey's test was used to determine any significant difference among cultivars at $p < 0.05$.

RESULTS AND DISCUSSION

Pulp color and internal quality parameters

The quality parameters (color of the pulp, TSS, TA and maturity index) of the fruit of the six citrus genotypes selected for this study are shown in Table 1. Color of the pulp (determined as the *a/b* Hunter ratio) and differences between cultivars of the same species were the main criteria for the selection of these genotypes. Accordingly, for mandarin fruit, Nadorcott

displayed a bright orange coloration (*a/b,* 0.50±0.01) more intense than the pale orange pigmentation of Clemenules (0.34±0.02). Nadorcott is a mandarin derived from the traditional Murcott mandarin in Morocco, characterized by a late harvest, excellent internal quality and the intense orange coloration of both peel and pulp (Nadori, 2004). By contrast, despite the excellent fruit quality and commercial relevance of Clemenules mandarin, its internal and external coloration is less intense than other mandarin cultivars (Barry et al., 2020). TSS was slightly higher in Nadorcott than in Clementine mandarin, indicating a late ripening, but the maturity index at the harvest time was similar in both genotypes (Table 1).

For grapefruits and oranges, a red-fleshed genotype for each species was selected. Star Ruby grapefruit exhibited a typical red coloration (1.04±0.13) in comparison with the characteristic pale yellowish of the pulp of Marsh grapefruit (0.01±0.03). However, internal maturity index was similar in both grapefruits (Table 1), indicating that the maturation process under Mediterranean conditions follows a similar pattern in both cultivars with the exception of fruit coloration (Alquézar et al., 2013). These two grapefruit varieties are very common and cultivated worldwide, and differences in pulp coloration have been long described, despite the important effect of the climatic conditions in grapefruit pigmentation (Xu et al., 2006; Alquézar et al., 2013; Barry el at., 2020). Valencia Ruby is the first red-fleshed mutant derived from the Valencia late sweet orange (Zacarias, 2017). This phenotypic difference is well exemplified by the reddish (0.60±0.03) coloration of the pulp in comparison with the pale-orange tint (0.06±0.01) of the Valencia late orange fruit harvested at that same maturity stage and environmental conditions. Valencia late is a classical orange cultivar cultivated worldwide with moderate to low coloration and carotenoid content (Fanciullino et al., 2008; Kato, 2012). The characteristic pulp pigmentation of the Valencia Ruby mutant resembles that of the red-fleshed Cara Cara, a mutant of Navel orange more intensively studied, with an altered carotenoid complement (Xu et al., 2008; Alquézar et al., 2008). However, parameters of internal maturation were statistically similar in both Valencia and Valencia Ruby oranges, indicating that other ripening processes in the mutant are not affected (Table 1).

Carotenoid content and composition

Differences in pulp coloration in fruits of the six citrus genotypes selected were assessed in relation to their carotenoid content and composition. By HPLC-DAD ten major carotenoids were identified and quantified in our samples. Total carotenoid content in the pulp showed a high variability among the six genotypes (Table 2). Then, carotenoid content in the pulp of Nadorcott was 3.2-times higher than that of Clemenules. Interestingly, carotenoids were substantially higher in the red-fleshed grapefruit and orange than in the corresponding

counterpart. In the white Marsh grapefruit, carotenoids were extremely low (< 0.1 mg 100 g^{-1} FW) in comparison with the red Star Ruby. Similarly, total carotenoids in the pulp of Valencia Ruby was near 20-times higher than in the pulp of Valencia late (Table 2).

A detailed analysis of the carotenoid complement in the pulp of the six varieties revealed significant differences that may account for the particular coloration of each genotype (Table 2). Thus, the pulp of Clemenules contained moderate amounts (between 0.25 and 0.31 mg 100 g^{-1} FW) of phytoene, phytofluene, β-cryptoxanthin and violaxanthin. It is remarkable that the pulp of Nadorcott mandarin contained 6- and 4-times more β-cryptoxanthin and violaxanthin, respectively, than that of Clemenules, whereas other carotenoids remained nearly similar. These major differences in these two xanthophylls (especially β-cryptoxanthin) may explain the deeper orange color observed in the pulp of Nadorcott compared with Clemenules (Table 1). β-Cryptoxanthin is the main carotenoid in the pulp and juice of mandarins, providing their intense orange coloration and being one of the main dietary sources of this compound (Ma et al., 2020). The higher accumulation of deep orange carotenoids, such as β-cryptoxanthin, is a distinctive feature of mandarin fruits and it has been proposed as a criterion to distinguish from sweet orange fruits (Goodner et al., 2001; Kato et al., 2004; Fanciullino et al., 2006). It is remarkable the high carotenoid and β-cryptoxanthin content detected in the pulp of Nadorcott mandarin. This mandarin is currently appreciated in the fresh-fruit market by the intense orange coloration of both peel and pulp, but a detailed analysis of its carotenoid content and composition has not been reported yet. In this study, we found that carotenoid content was in the range reported for other mandarin hybrids, such as its parental Murcott (Alquézar et al., 2008; Petry et al., 2019). Moreover, the content of β-cryptoxanthin in Nadorcott pulp account for about 40% of total carotenoids, while in Clemenules is 21% (Table 2), therefore, the high content and proportion of β-cryptoxanthin in Nadorcott may explain the intense orange coloration of its pulp.

Star Ruby was selected in this study as a pigmented grapefruit by the distinctive red coloration of the flesh due to the large accumulation of lycopene compared with other red-pigmented grapefruit cultivars (Xu et al. 2006; Alquézar et al., 2013). Analysis of carotenoids profile in the pulp of the Star Ruby revealed that the carotenes phytoene (31% of total carotenoids), lycopene (28.5%), β-carotene (22%) and phytofluene (12%) were the most abundant. By contrast, the pulp of the white Marsh was almost devoid of carotenoids, with only minor amounts of phytoene, phytofluene and xanthophylls (Table 2). These results are in good agreement with those reported in other studies for the same grapefruit varieties (Xu et al., 2006; Fanciullino et al., 2008, Alquézar et al. 2008, 2013) and corroborate the motion that the usual accumulation of lycopene in red grapefruits appears to be the result of a partial

blockage in the cyclization of lycopene leading an accumulation of earlier linear carotenes (Alquézar et al., 2008).

Comparison of carotenoid profiling in the pulp of Valencia late orange and its red-fleshed Valencia Ruby mutant revealed a completely altered carotenoid content and composition (Table 2). First, total carotenoids were 20-times higher in Valencia Ruby than in Valencia late; second, Valencia late accumulated almost exclusively the β,β-xantophylls violaxanthin (44.2%), anteraxanthin (16.3) and β-cryptoxanthin (7%); and third, Valencia Ruby accumulated large amounts of linear carotenes phytoene (80.6%), phytofuene (6%) and lycopene (5.2%), whereas total xanthophylls were less than 3% of total carotenoids (Table 2). This unusual carotenoid profiling in the pulp of Valencia Ruby and the accumulation of lycopene resembles that reported for the two other red-fleshed oranges, Hong Anliu and Cara Cara, so far characterized (Liu et al., 2007; Alquézar et al., 2008; Rodrigo et al., 2015; Lu et a., 2017). It is interesting to remark that the concentration of lycopene reported in the pulp of mature fruit of these mutants is about 0.5 to 1.0 mg 100 g^{-1} FW and in the same range of that found in the present study for Valencia Ruby (Xu et al., 2006; Alquézar et al., 2008; Rodrigo et al., 2015). However, total carotenoids in Valencia Ruby were substantial higher, mainly due to the accumulation of the early linear carotenes phytoene and phytofluene that reached concentrations extremely high (13.31 and 1.84 mg 100 g^{-1} FW, respectively) (Table 2). To our knowledge, this is the first report of the analysis of carotenoids in a red-fleshed mutant of Valencia orange and, it is worth to remark that such large amount of phytoene in its flesh is the highest so far reported for a citrus fruit (Alquézar et al., 2008; Rodrigo et al., 2003, 2019). Since accumulation of significant amounts of phytoene is not common in fruits of other species (Alquézar et al., 2008) and that important health-related benefits have been assigned to this carotene (Rao and Rao et al., 2007; Meléndez-Martínez et al., 2018, 2019), Valencia Ruby is an useful and promising orange cultivar in order to provide fresh fruit or derived products with added value and potential health benefits.

Vitamin C content

Vitamin C (Vit C) is one of the most important antioxidant compounds in plant cells and a major contributor to the health-related benefits attributed to citrus fruit (Alós et al., 2014, Zou et al., 2016). The concentration of Vit C in the pulp of the six cultivars selected for the current study were between 19.04 and 45.29 mg 100 g^{-1} FW (Figure 2). Valencia Ruby and Valencia late sweet oranges, and Marsh grapefruit showed the highest Vit C content followed by Star Ruby grapefruit and Clemenules mandarin. Nadorcott mandarin was the cultivar with the lowest vitamin C concentration (45% lower than Clemenules mandarin) (Figure 2). These

results are in agreement and in the same range than that reported by other authors in different *Citrus* species and varieties (Cano et al., 2008; Martí et al., 2009; Alos et al., 2014; Kafkas et al., 2011). Conclusions from these studies indicated that Vit C concentration in the fruit appear to be related to the genotype and/or to the stage of development (Alós et al., 2014). Moreover, oranges contain the highest Vit C concentrations among the *Citrus* genus, followed by lemons, grapefruits and mandarins, with a higher variability among mandarins than in other species (Martí et al., 2009). Interestingly, comparison of Vit C content in several mandarin varieties indicated that Murcott (Cano et al., 2008) and Nadorcott mandarin (Nardello et al., 2018) contained about half of that of other Clementine mandarins, similarly to the differences obtained in the current work (Figure 2). It is important to note that other studies have detected between 39% and 15% reduction in Vit C concentration in the juice of the lycopene-accumulating sweet orange Cara Cara compared to its parental Navel juice (De Ancos et al., 2020; Kafkas et al., 2011). However, the Vit C content in the red-fleshed Valencia Ruby is similar to Valencia, and are the highest values among the genotypes analyzed in this study.

Antioxidant capacity in hydrophilic and lipophilic pulp extracts of citrus cultivars

In order to evaluate the contribution of carotenoids and Vit C content to the antioxidant capacity of citrus fruit, the antioxidant capacity of the pulp of the six citrus varieties was analyzed. The antioxidant capacity of the hydrophilic (HAC) fraction was evaluated by two methods, DPPH and ABTS scavenging assays, while the antioxidant capacity of lipophilic (LAC) fraction was determined by ABTS. The HAC of extracts showed a significant variability among cultivars (Figure 3). By the characteristics of each assay, values of the HAC obtained by ABTS were quantitatively higher than those of DPPH, but results of the different varieties were consistent between both assays. Comparison of HAC between cultivars of the same species revealed that Nadorcott and Star Ruby had lower capacity than Clemenules mandarin and Marsh grapefruit, respectively, and in sweet oranges only a minor reduction in HAC in Valencia late pulp with respect to Valencia Ruby was detected by the ABTS assay (Figure 3). Evaluation of antioxidant capacity in different food matrix are usually performed by several methods, such as ABTS, DPPH, FRAP (Ferric Reducing Antioxidant Power), and ORAC (Oxygen Radical Absorbance Capacity) (Müller et al., 2011; Apak et al., 2013, 2016). The ABTS assay has the advantage of being used over a wide range of pH, hence, it is more useful to study and compare foods at different pH. Moreover, the ABTS assay requires a shorter reaction time compared to the DPPH assay. In addition, ABTS is soluble in polar and non-polar solvents and can be therefore used to assess both water- and lipid-soluble antioxidants (Yoo and Moon,

2016). The scavenging ability of the different bioactive compounds varies widely by the mode of action of each radical (Gironés-Vilaplana et al., 2014), but, in general, the values of antioxidant capacity obtained by ABTS use to be higher than those by DPPH.

Analysis of the HAC in the pulp of the six citrus varieties analyzed revealed a parallelism with the profiling of Vit C content (Figure 2). The correlation analysis between HAC and Vit C for the six citrus varieties studied indicates a high correlation with r^2 values of 0.85 and 0.91 for the DPPH and ABTS assays, respectively. These results point out that in the pulp of mandarins, grapefruits and sweet oranges the concentration of Vit C is closely related to their hydrophilic antioxidant activity. Our results highly support previous observations indicating that Vit C is a major contributor to the total antioxidant capacity in fruits and juices of many *Citrus* species and varieties (Sicari et al., 2016; Yoo and Moon, 2016; Elkhatim et al., 2018; Sánchez-Moreno et al., 2002, 2003; Martí et al., 2009).

By contrast, a virtual absence of correlation between the hydrophilic activity by both DPPH and ABTS assays, and carotenoid content in the six varieties was obtained (r^2= 0.01, r^2= 0.13, respectively). These results are not unexpected, since the extraction for the HAC determination was performed with polar solvents and only a minor fraction, if any, of the carotenoids present in the tissue would be extracted. Guddadarangavvanahally et al. (2008) have also discussed the influence of the extraction solvents in the analysis of the antioxidant capacity of grapefruit and oranges. Then, special attention should be taken in the interpretation of the potential contribution of carotenoids to the antioxidant capacity of foods or plant tissues extracts.

In order to explore more in detail, the involvement of the different carotenoid content and composition in the antioxidant activity of the pulp, we independently extracted and determined the scavenging capacity of the lipophilic fraction by ABTS assay. The lipophilic extracts of the pulp of both sweet orange cultivars showed the highest LAC (Figure 4). Comparison of LAC in grapefruit and mandarin varieties revealed that the red Star Ruby had higher capacity than Marsh, however, no significant differences were detected between Nadorcott and Clemenules (Figure 4). Together, the antioxidant capacity of the analyzed citrus pulp lipophilic extracts showed a weak positive correlation (r^2=0.40) with total carotenoids content. Moreover, we did not observe either significant correlation between individual carotenoids and LAC (data not shown). Carotenoids are fat-soluble compounds and have potential antioxidant properties because they quench singlet oxygen (Stahl and Sies, 2003). Nevertheless, the contribution of carotenoids to the antioxidant capacity of citrus fruit is still a matter of debate, since controversial results have been obtained (Arnao et al., 2001; Sánchez-Moreno et al., 2003; De Ancos et al., 2002, 2020). Despite the antioxidant activity in the

lipophilic fraction has received little attention (Cano et al., 2000, 2004; Arnao et al., 2001; Rodríguez-Amaya et al., 2010), our results indicate that other recognized antioxidant compounds (such as tocopherols), in addition to carotenoids, may also significantly contribute to the LAC of the pulp of Citrus fruit (Rodríguez-Amaya et al., 2010; Arnao et al., 2001; Sánchez-Moreno et al., 2003).

Comparison of HAC and LAC assayed by ABTS indicated that the former was 5- to 10-times higher than LAC (Figure 3 and 4). This result is similar to that reported in other studies and reinforces the conclusion that hydrophilic compounds are the major contributors to the total antioxidant capacity of citrus fruit pulp (Arnao et al., 2001; Sánchez-Moreno et al., 2003; Cano et al., 2004; Wu et al., 2009; Yoo and Moon, 2016).

Analysis of the potential contribution of vitamin C and carotenoids to total antioxidant capacity

Total antioxidant capacity in a food matrix is the combined activity of different antioxidants compounds such as carotenoids, vitamin C, polyphenols, tocopherols and others (Patil et al., 2017). One of the main goals of this study was to evaluate the specific contribution of carotenoids and Vit C to the lipophilic and hydrophilic antioxidant capacity, respectively, using the genetic diversity in pulp pigmentation and in the carotenoid complement found in *Citrus* species and cultivars. Carotenoid analysis in the pulp of the six varieties selected in this study revealed important differences in the total content but also an enrichment in specific carotenoids: lycopene in Star Ruby or Valencia Ruby, phytoene in Valencia Ruby, or β–cryptoxanthin in Nadorcott mandarin (Table 2). To estimate the contribution of carotenoids and Vit C of each variety to the LAC and HAC, respectively, we used the relative antioxidant capacity value of pure carotenoids and vitamin C described by Müller et al. (2011). Then, total antioxidant capacity is calculated as the sum of the relative contribution of each specific carotenoid or xanthophyll (expressed as % of the total) present in the pulp of the citrus fruit. This approach showed that Vit C contributed to the HAC between 18% in Nadorcott mandarin and 35% in Valencia orange (Figure 5A). These values indicate that other components (such as phenolic compounds) are greater contributors than Vit C to the HAC of the pulp of citrus fruit. Similar results have been also obtained in fruits of different citrus cultivars (Yoo and Moon, 2016; Shin, 2012 and Sicari et al., 2016), but other studies estimated that Vit C is the predominant antioxidant in *Citrus* (Shin, 2012; Xu et al., 2008; Yoo et al., 2004, Sánchez-Moreno et al., 2003).

On the other hand, the relative contribution of carotenoids to LAC showed a wide variability among genotypes (Figure 5B). As expected, the cultivars with high total carotenoids

content exhibited high contribution to LAC: Valencia Ruby orange and Nadorcott mandarin (77%) followed by Star Ruby (41%), Clemenules (20%) and Valencia late (5%), while that of Marsh grapefruit was almost negligible (Figure 5B). However, this sequence did not follow the carotenoid content of each variety (Table 2). This discrepancy indicates that other lipophilic compounds (as tocopherols) are likely contributing to the LAC and that synergistic effects among them may modify total antioxidant activity (Rodríguez-Amaya, 2010). It is worth to mention that the varieties with higher contribution of carotenoids to LAC are Valencia Ruby sweet orange, which contains lycopene and also an extremely high concentration of phytoene, both carotenes have been postulated to have antioxidant properties (Bailey et al., 2015; Meléndez-Martínez et al., 2018); and the mandarin Nadorcott, with the largest content of β–cryptoxanthin, a xanthophyll with demonstrated protection against oxidative stress by using an *in vivo* assay (Llopis et al., 2019). Then, it is likely that the contribution of carotenoids to LAC is not only dependent of their total amount but also of the concentration of specific carotenoids, and thus, our results may explain the lack of consistency between carotenoid content and the antioxidant capacity observed in different studies in citrus fruit (reviewed by Zou et al., 2016).

In conclusion, *in vitro* antioxidant assays may be useful indicators to estimate the healthy properties of the edible part of fruits. However, the lack of standardization, the particular features of the different methods and the ability of compounds to scavenge/quench different radicals, may generate discrepancies and over- and underestimate the specific contribution of the different hydro and lipophilic components of a food sample (Rodríguez-Amaya et al., 2010; Apak et al., 2013; 2016). In this work, we have used two antioxidant assays to determine HAC and LAC in the pulp of six citrus varieties with contrasting coloration and carotenoid content and composition. Results indicated a positive correlation between Vit C content and HAC, while total carotenoids did not parallel LAC. The contribution of Vit C was estimate to be 15-30% of the total HAC. Carotenoids had a very variable contribution to LAC, being the highest that of Valencia Ruby orange, with large concentrations of lycopene and phytoene, and Nadorcott mandarin, with high β–cryptoxanthin content.

ACKNOWLEDGEMENTS

JZ is the recipient of a FDEGENT/2018/007 predoctoral scholarship from Generalitat Valenciana (Spain) and FR is the recipient of a predoctoral scholarship from the Agencia Nacional de Investigación e Innovación (ANII, Uruguay). This work was supported by a research grants AGL2015-70218 and RTI2018-095131-B-I00 of the Ministry of Science, Innovation and

Universities (Spanish Government). Authors are members of the CaRed Excellence Network (BIO2015-71703-REDT and BIO2017-90877-REDT) and European COST Action CA15113 EUROCAROTEN. Authors acknowledged the Fundación ANECOOP and the Citrus Germoplasm Bank of IVIA (Generalitat Valenciana) for the provision of fruits and the Department of Food Technology, University Miguel Hernández for the technical advice in the antioxidant assays.

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Table 1. Color index (*a/b* Hunter ratio) of the pulp, and total soluble solids (TSS, º Brix), total acidity (TA, mg citric acid 100 ml^{-1}) and maturity index (TSS/TA) of the juice of fruit of the six Citrus genotypes used in this study.

Data are expressed as mean ± standard deviation (n=3).

Different letters in the same column indicate significant differences ($p<0.05$) by Tukey's test.

Cultivar	**Pulp color** (*a/b* Hunter)	**TSS** (ºBrix)	**TA** (mg citric acid 100 ml^{-1})	**IM** (TSS/TA)
Clemenules mandarin	0.34±0.02d	9.83±0.06d	0.53±0.01d	18.59±0.92a
Nadorcott mandarin	0.50±0.01c	10.90±0.20c	0.70±0.20d	15.57±0.50a
Marsh grapefruit	0.01±0.03f	11.60±0.01b	1.88±0.01b	6.19±0.60c
Star Ruby grapefruit	1.04±.013a	12.27±0.06a	2.24±0.02a	5.48±0.40c
Valencia late orange	0.06±0.01	11.50±0.10b	1.00±0.10c	11.50±0.90b
Valencia Ruby orange	0.60±0.03b	10.90±0.10c	1.10±0.20c	9.90±1.10b

Table 2: Carotenoid content and composition (mg 100 g^{-1} FW, fresh weight) in the pulp of six Citrus cultivars. Data are expressed as mean ± SD. Tr, traces; nd, non detected.

[α] Total carotenoids are the sum of the main identified and quantified carotenoids.

For total carotenoids, different letters indicate significant differences ($p<0.05$) by t-Student test.

	Mandarins		Grapefruits		Oranges	
Carotenoid (mg 100 g^{-1} FW)	**Clemenules**	**Nadorcott**	**Marsh**	**Star Ruby**	**Valencia late**	**Valencia Ruby**
Phytoene	0.26±0.03	0.27±0.14	0.03±0.01	0.81±0.02	0.1±0.01	13.31±0.11
Phytofluene	0.31±0.27	0.28±0.13	0.01±0.01	0.32±0.01	0.02±0.01	1.84±0.36
ζ-Carotene	0.07±0.01	0.26±0.13	nd	0.03±0.01	0.03±0.01	0.06±0.01
Lycopene	nd	nd	nd	0.74±0.08	nd	0.86±0.02
β-Carotene	tr	0.24±0.12	nd	0.57±0.02	nd	0.04±0.01
β-Cryptoxanthin	0.31 ±0.03	1.83±0.50	nd	0.01±0.01	0.06±0.02	0.03±0.01
Zeaxanthin	0.04±0.01	0.09±0.02	nd	0.03±0.03	0.07±0.02	0.06±0.01
Anteraxanthin	0.10±0.01	0.32±0.02	0.01±0.01	0.02±0.02	0.14±0.01	0.11±0.03
Luteoxanthin	0.02±0.01	0.11±0.03	nd	nd	0.06±0.02	0.02±0.01
Violaxanthin	0.25±0.01	1.00±0.04	0.01±0.01	nd	0.38±0.02	0.23±0.01
Total carotenoids[α]	**1.44±0.26d**	**4.69±1.06b**	**0.06±0.01f**	**2.60±0.15c**	**0.86±0.03e**	**16.51±0.28a**

Figures legends

Figure 1. Internal appearance of mature fruit of the *Citrus* varieties used in this study: Clemenules (*Citrus clementina*) and Nadorcott (*Citrus reticulata*) mandarins, Valencia late and Valencia Ruby oranges (*Citrus sinensis*), and Marsh and Star Ruby grapefruits (*Citrus paradisi*).

Figure 2. Vitamin C concentration (mg 100 g^{-1} fresh weight, FW) in pulp of mature fruits of six Citrus cultivars; CM, Clemenules mandarin; ND, Nadorcott mandarin; MS, Marsh grapefruit; SR, Star Ruby grapefruit; VL, Valencia late orange; VR, Ruby Valencia orange. Data are expressed as mean ± SD (n=3). Different letters indicate significant differences ($p<0.05$) by Tukey's test.

Figure 3. Antioxidant capacity of the hydrophilic fraction, assayed by DPPH and ABTS (mg of Trolox equivalents 100 g^{-1} FW) in the pulp of fruits of six Citrus cultivars. CM, Clemenules mandarin; ND, Nadorcott mandarin; MS, Marsh grapefruit; SR, Star Ruby grapefruit; VL, Valencia late orange; VR, Ruby Valencia orange Small and capital letters indicate significant differences ($p<0.05$) for DPPH and ABTS, respectively, by Tukey's test.

Figure 4. Antioxidant capacity of the lipophilic fraction determined by ABTS (mg of Trolox equivalents 100 g^{-1} FW) in the pulp of fruits of six Citrus cultivars. CM, Clemenules mandarin; ND, Nadorcott mandarin; MS, Marsh grapefruit; SR, Star Ruby grapefruit; VL, Valencia late orange; VR, Ruby Valencia orange Different letters indicate significantly differences ($p<0.05$) by Tukey's test.

Figure 5. Percentage of the contribution of vitamin C and other compounds to the total hydrophilic antioxidant capacity **(A)**; and of total carotenoids and other compounds to the total lipophilic antioxidant capacity **(B)** in the pulp of fruits of six Citrus cultivars. CM, Clemenules mandarin; ND, Nadorcott mandarin; MS, Marsh grapefruit; SR, Star Ruby grapefruit; VL, Valencia late orange; VR, Ruby Valencia orange

Figure 1

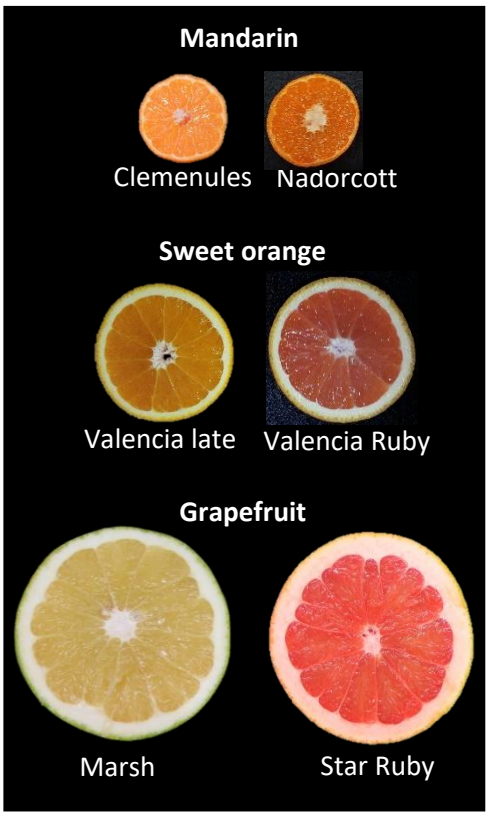

Mandarin
Clemenules
Nadorcott
Sweet orange
Valencia late
Valencia Ruby
Grapefruit
Marsh
Star Ruby

Figure 2

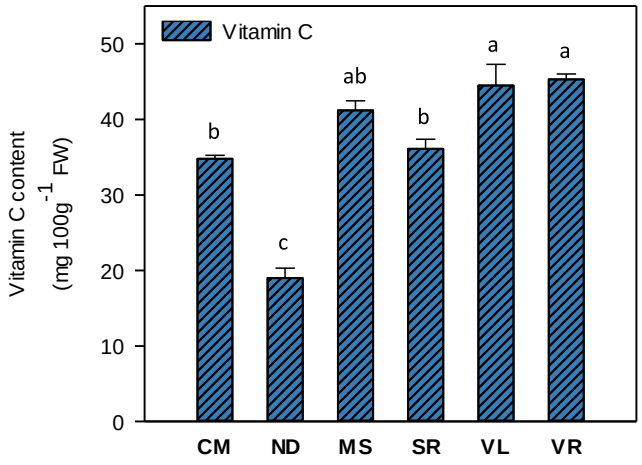

Vitamin C
Vitamin C content
(mg 100g -1 FW)
50
40
30
20
10
0
b
c
ab
b
a
a
CM
ND
MS
SR
VL
VR

Figure 3

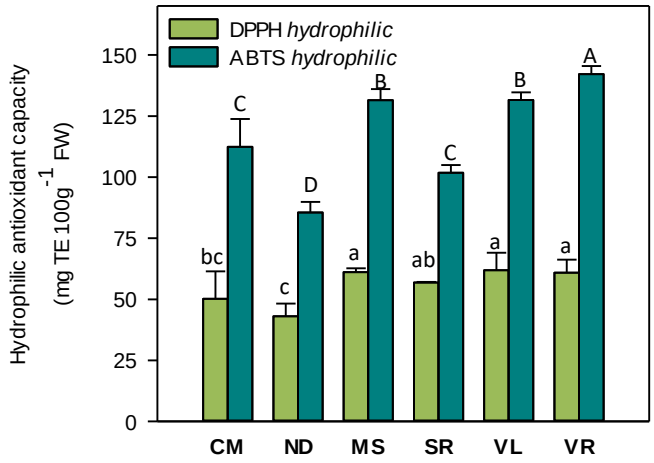
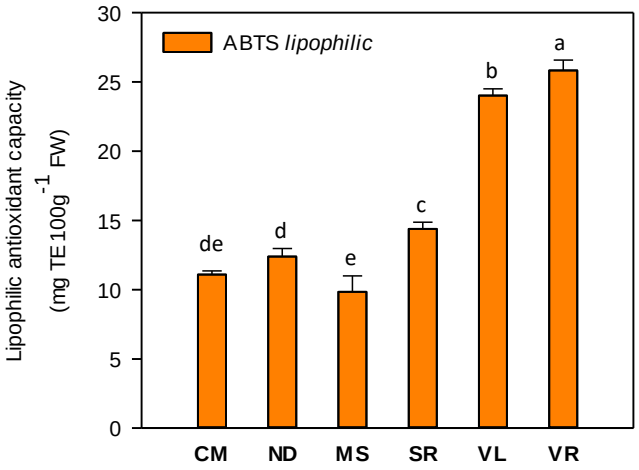

DPPH hydrophilic
ABTS hydrophilic
Hydrophilic antioxidant capacity
(mg TE 100g -1 FW)
150
125
100
75
50
25
0
bc
C
c
D
a
B
ab
C
a
B
a
A
CM
ND
MS
SR
VL
VR

Figure 4


ABTS *lipophilic*
Lipophilic antioxidant capacity
(mg TE 100g -1 FW)
30
25
20
15
10
5
0
de
d
e
c
b
a
CM
ND
MS
SR
VL
VR